# APPENDIX I

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# Differential enhancement of Cry2A versus Cry11A yields in *Bacillus thuringiensis* by use of the *cry3A* STAB mRNA sequence

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**Abstract**

Previously we demonstrated that the yield of Cry3A (70 kDa) can be increased as much as 10-fold when *cry3A* including its upstream STAB-SD mRNA stabilizing sequence is expressed in *Bacillus thuringiensis* under the control of *cyt1A* promoters. To determine whether the *cyt1A* promoters/STAB-SD combination (*cyt1A*P/STAB) has broader applicability, we used it to synthesize two other Cry endotoxins in the 70-kDa mass range, Cry2A and Cry11A. Combination of *cyt1A*P/STAB with *orfs 2* and *3* of the *cry2A* operon yielded about 4.4-fold the amount of Cry2A obtained with the wild-type *cry2A* operon. The yield of Cry11A obtained with a construct that contained the *cyt1A*P/STAB, *cry11A* and the 20-kDa protein gene was 1.3-fold the amount obtained with a construct similar to the wild-type operon. These results demonstrate that the *cyt1A*P/STAB combination can enhance synthesis of different Cry proteins significantly, but that the level of enhancement varies with the specific protein synthesized. 

*Keywords:* Cry protein synthesis; Shine-Dalgarno sequence; mRNA stability; *Bacillus thuringiensis*

## 1. Introduction

The bacterium *Bacillus thuringiensis* produces insecticidal proteins during sporulation that serve as the active ingredients of commercial bacterial insecticides, and are used increasingly in insecticidal transgenic plants [1]. In *B. thuringiensis*, these proteins form crystals, with the most common types being composed of Cryl proteins of about 135 kDa. These are primarily toxic to lepidopterous insects, and consist of a N-terminal half containing the toxic portion of the molecule, released after ingestion by insect midgut proteases, and a C-terminal half important to crystallization [2,3]. In addition to 135-kDa proteins, Cry proteins of 65–70 kDa are known which correspond to the N-terminal half of the 135-kDa Cry type. Examples include Cry2A toxic to lepidopterous and dipterous insects, Cry3A toxic to coleopterous insects, and Cry11A toxic to certain dipterous insects.

An interesting characteristic of Cry synthesis is that the 135-kDa protein type forms a large crystal in each cell comprising as much as 35% of its dry weight, whereas the 65-kDa proteins form a much smaller crystal ranging from less than a fifth to a third the size of Cryl crystals [4–6]. Some increase

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in 65-kDa protein crystal size is obtained when their genes are expressed in the absence of other *cry* genes that compete for transcription factors, energy sources, and amino acids [7–10]. However, the size of these crystals is typically less than half that of Cryl crystals produced under similar conditions.

Crystals formed by the 65-kDa proteins are almost pure toxin, and thus increasing the amount of these produced per cell is a strategy being used to improve the efficacy of insecticidal bacteria. Knowledge of mechanisms that affect Cry toxin synthesis has been used to obtain substantial increases in 65-kDa protein yields per cell. For example, the 20-kDa chaperone-like protein encoded by *cryl1A orf3* enhances Cyt1A and Cryl1A synthesis [10,11]. Placing the 20-kDa protein gene under the control of *cry1Ac* promoters increased yields of Cryl1A and Cry2A by about 1.5-fold [9,10]. Strong and multiple promoters also contribute substantially to toxin synthesis, and use of *cyt1A* promoters to drive expression of *cry3A* increased Cry3A yield two-fold in comparison to the wild-type strain [12]. Transcript stability is another factor important to Cry synthesis, and the most substantial increases in synthesis of the 65-kDa protein type have been obtained using the Shine-Dalgarno mRNA stabilizing sequence (STAB-SD) located in the 5′ region of the *cry3A* gene [13]. Expression of a construct containing this sequence and *cry3A* under its own promoter, active during the stationary growth phase, in an asporogenic strain of *B. thuringiensis*, resulted in five to six-fold increases of Cry3A [14]. Even higher yields of about 10-fold were obtained by driving the expression of the STAB-SD *cry3A* construct with sporulation-dependent *cyt1A* promoters [12]. Cry3A increases with constructs that contained the *cyt1A* promoters but lacked the STAB-SD sequence were five-fold less than those that contained this sequence, demonstrating that the STAB-SD sequence was the key factor responsible for the substantial increase in Cry3A.

The marked increase in Cry3A yield obtained with *cyt1A* promoters combined with the STAB-SD sequence suggested that this combination might be useful for increasing yields of other toxins of 65–70 kDa. Thus, we evaluated this combination for the production of Cry2A and Cryl1A. Here we show that this combination can increase Cry2A yield as much as 4.4-fold, and Cryl1A yield by about 1.3 fold in comparison to expression systems which lacked the STAB-SD sequence.

## 2. Materials and methods

### 2.1. Bacterial strains, genes and plasmids

The *Escherichia coli-B. thuringiensis* shuttle expression vector pHT3101 [15] was used to make and amplify all *cry2Aa* and *cryl1Aa* constructs in *E. coli* DH5α. The plasmid pCL-92 containing the full-length *cry2Aa* operon was provided by Dr. W.J. Moar (Department of Entomology, Auburn University, Auburn, AL, USA). The 4-kb *Eco*RI-*Hin*dIII fragment bearing the *cry2Aa* operon in pCL-92 was cloned into the same site in pBlueScript (SK+) (Stratagene) to generate pEH-2. The *cryl1Aa* gene was obtained from *B. thuringiensis* subsp. *israelensis* [10]. Plasmids used as controls were pDBF69 for Cry2Aa production [9] and pWF53 for Cryl1Aa production[10]. All constructs were expressed in the

Table 1
Primers used for PCR construction and amplification of *cyt1A*P/STAB-SD constructs for synthesis of Cry2A and Cryl1A

| Primer | Sequence (5′→3′)[a] |
|---|---|
| STAB-1 | G**GAATTC**GATTTCAAATTTTCCAAACTTAAA |
| STAB-2 | GC**TCTAGA**CTTTCTTATCATAATACATAATTTTCA |
| Cry2A-1 | GC**TCTAGA**AAGGAGGAATTTTATATGAATAATGTA |
| Cry2A-2 | GC**TCTAGA**ATAGGAGGAAAAGATTTTATGCTAAAA |
| Cry2A-3 | GC**TCTAGA**AAGCTTTAGGTTAACTTGAAATGATTTCTCCC |
| Cryl1A-1 | GC**TCTAGA**AAAGGTGGAATGAATTATATGGAA |
| Cryl1A-2 | GC**TCTAGA**ATGATTTTCTACAATTACAT |
| Cryl1A-3 | GC**TCTAGA**TTTAGGTCTTTAAAAATTAGAACCAATAATTTA |

[a]Restriction endonuclease cleavage sites for *Eco*RI and *Xba*I are in bold face; ATG codons are underlined.

acrystalliferous 4Q7 strain of *B. thuringiensis* subsp. *israelensis* obtained from the *Bacillus* Stock Center at Ohio State University, Columbus, OH, USA. Hereafter, *cry2Aa* is referred to as *cry2A*, *cry11Aa* as *cry11A*, and *cyt1Aa* as *cyt1A*.

### 2.2. Expression vector construction

Oligomers used to make the various constructs for expression of *cry2A* and *cry11A* with *cyt1A* promoters and the STAB-SD sequence are listed in Table 1. The specific constructs used to express *cry2A* and *cry11A* are illustrated schematically in Fig. 1. The plasmid pPF-CS containing *cyt1A* promoters and the STAB-SD sequence was obtained by inserting a 660-bp product, obtained by polymerase chain reaction (PCR) using primers STAB-1 and STAB-2, into the *Eco*RI and *Xba*I site of pHT3101 (Fig. 1A). For *cry2A* synthesis using pPF-CS, two constructs were tested, pPFT2As, containing the *cry2A* gene, and pPFT2Asf, containing the *cry2A orf2* (open reading frame 2) and the *cry2A* gene. The *cry2A* operon ORF2 protein has been shown to be essential for proper formation of the Cry2A inclusion [9]. The above *cry2A* fragments were obtained by PCR from pEH-2 using the primers cry2A-1/cry2A-3 and cry2A-2/cry2A-3. PCR products from these reactions were inserted into the *Xba*I site of pPF-CS to create, respectively, pPFT2As (*cyt1A*P/STAB+*cry2A*) and pPFT2Asf (*cyt1A*P/STAB+*cry2A orf2*+*cry2A*). To observe the effect of STAB-SD on the crystal protein production, the PCR product used to create pPFT2Asf was inserted into same site of pHTCytA that has *cyt1A* promoters without STAB-SD sequence (pPFT2Af) [12]. For synthesis of Cry11A using *cyt1A* promoters/STAB-SD system, two different constructs were tested, pPFT11As containing the *cry11A* gene, and pPFT11Ast containing *cry11A* and the 20-kDa protein gene. DNA fragments containing these genes were obtained by PCR from *B. thuringiensis* subsp. *israelensis* 4Q5 plasmid DNA as a template and the primers cry11A-1/cry11A-2 (for *cry11A*) and cry11A-1/cry11A-3 (for *cry11A*+20-kDa gene). These fragments were then cloned separately into the *Xba*I site in pPF-CS to generate, respectively, pPFT11As and pPFT11Ast. A PCR fragment used to construct pPFT11Ast was inserted into the *Xba*I site of pHTCytA to generate pPFT11At that lacked the STAB-SD sequence. All constructs were confirmed by restriction enzyme analysis and DNA sequencing.

### 2.3. Polymerase chain reaction

PCR was performed with the Expand™ Long Template PCR System (Boehringer Mannheim, GmbH, Germany) for 30 cycles as follows: 94°C for 1 min, 55°C for 1 min and 68°C for 3 min.

### 2.4. Bacterial transformation and Cry protein quantification

Bacterial transformation was essentially as described previously [12]. To determine the amount of crystal protein produced by the various constructs in *B. thuringiensis* cells, each strain was grown at 30°C in 50 ml of Nutrient Broth plus Glucose (NBG) medium in 250-ml flasks shaken at 250 rpm for 5 days. Cultures were then harvested at 6500 rpm for 15 min, and after drying under vacuum, the pellets were weighed for bioassay. The relative amount of Cry protein produced per unit of medium by each construct was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sporulated cultures of transformed and wild-type cells, 200 μl per culture, were disrupted in 5×Laemmli sample buffer, and boiled for 5 min. Proteins were separated by subjecting 20 μl samples to electrophoresis through a 10% gel [16]. The protein bands were scanned by the GAS 4000 gel documentation system (Evergene). The amount of protein in each band was quantified with ImageQuant 4.1 densitometry software (Molecular Dynamics, Sunnyvale, CA, USA) as described previously [12]. Relative toxin yields were also assessed on a per spore basis by quantifying the amount of toxin produced by an equal number of spores, based on final spore counts, transformed with each construct. The quantification of relative yields was by SDS-PAGE and gel scanning as described above.

### 2.5. Estimation of cell viability and Cry crystal purification

To determine the number of spores formed per ml of medium, cells were grown in 50 ml of NBG in

**A**

*Eco*RI
GAATTCTATT TTCGATTTCA AATTTTCCAA ACTTAAATAT GATTGAATGC 50

CTGAGAAAGG TAATAGAGAT GTTTTAGTTT ATTATGAAGT ATTAGGGGCG 100

TCTTTTAAAT TCAATCTATC AATTTGTGAA ATATATTACT CAAAACCCAA 150

TACCATTCTA AAACTTATTC AAAATATATA TTGCTTTAAA AGAGCATACA 200

TACTAAAAAA ACAGGCATCT TTCGAACTAT AGCGCATAGA ATACTACGGT 250
-35 -10

GAATCAAAAA CAAATAAAAT TTAGGAGGTA TATTCAAGTA TACAAAAAAA 300

CTTTAGTGTG AGGGGATTTA GATAAAAAGT ATTCGTTATC CTTATAAATT 350

AATTCTTAAA CATGCACCAA TGTATACATT AAATAATATT ATGTGAATTA 400
-35 -10

AGTCTATCAA TTTAATTTAT TATGTTACTT TATATTTGAT TAATAATTGC 450

AAGTTTAAAA TCATAATTTA ATGTTGAAAG GCCACTATTC TAATTAACTT 500

AAGGAGTTGT TTATTTGAGC TCGGTACCCG GGGATAATCT TGAAAGGAGG 550
STAB-SD

GATGCCTAAA AACGAAGAAC ATTAAAAACA TATATTTGCA CCGTCTAATG 600

GATTTATGAA AAATCATTTT ATCAGTTTGA AAATTATGTA TTATGATAAG 650

*Xba*I
AAAGTCTAGA 660

**B**

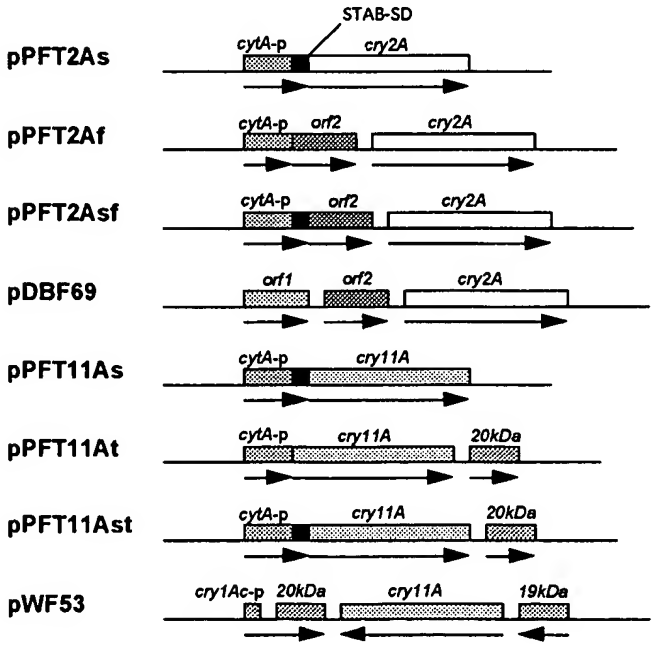


Fig. 1. Upstream region and schematic illustration of constructs used to synthesize Cry2*A* and Cry11*A*. A: Nucleotide sequence of the 660-bp fragment containing *cyt1A* promoters combined with STAB-SD sequence. Sigma E-like and sigma K-like promoters of *cyt1A* are shown, respectively, in boxes or underlined. The STAB-SD sequence is highlighted as a black box. B: Schematic of the different constructs used to synthesize Cry2A or Cry11A. Arrows indicate orientation of genes. Constructs pDBF69 and pWF53 used, respectively, the wild-type *cry2A* and *cry11A* promoters to drive expression.

250-ml flasks shaken at 250 rpm for 5 days at 30°C. The protocol used for spore counts was that described previously [12]. Data were analyzed with the Super ANOVA program (Abacus Concepts, Berkeley, CA, USA). Cry2A and Cry11A crystals were purified on NaBr gradients, using a method described previously [5].

### 2.6. Bioassays

The toxicity of purified Cry2A or Cry11A crystals was determined by bioassays employing second instars of *T. ni* or first instars of *A. aegypti*. For *T. ni*, the diet incorporation bioassay was used [17]. Lepidopteran larvae in all tests were obtained from eggs (Entopath) and maintained on artificial diet [4] at 27 ± 1°C with a photoperiod of 16:8 (L:D). Five to seven concentrations were tested per construct. Each concentration was added to 0.5 g artificial diet. The toxin was mixed into the diet with a vortex mixer for 1 min. Approximately 300 μl was then poured into each well of a 24-well microtiter plate (Falcon, no. 3047). After the diet solidified, two second instars were placed in each well. Plates were covered with moistened filter paper and the lids replaced. Twenty-five larvae were evaluated per concentration and mortality was assessed at day 7 for all treatments.

For bioassays against *A. aegypti*, eggs from a laboratory colony were hatched by incubation overnight in distilled water at 28 ± 1°C. One first instar (17–18 h old) was placed in each well of a 96-well tissue culture plate (Corning, no. 25860). Six to seven concentrations of the test Cry protein were used per construct. Distilled water was added and then crystal suspensions were added so that each well contained approximately 200 μl per well. The plates were covered and kept in an incubator. Mortality data were recorded after 24 h and analyzed using Probit analysis (SAS Institute).

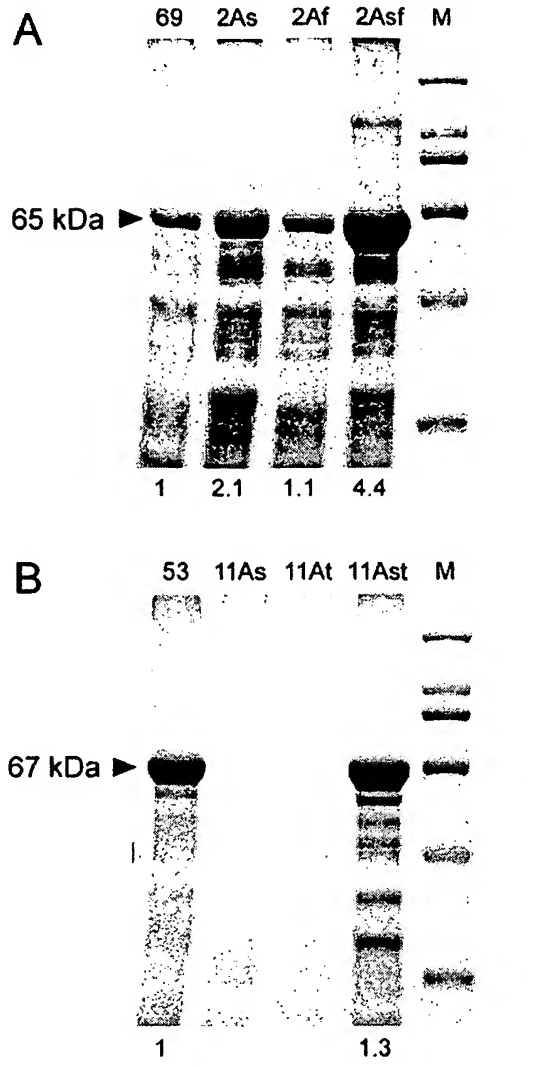


Fig. 2. SDS-PAGE analysis of Cry2A and Cry11A yields produced by *B. thuringiensis* subsp. *israelensis* 4Q7 strains expressing different constructs. The relative amount of crystal protein produced by each strain is indicated at the bottom of the gel. A: Strains producing Cry2A. Lane 1, pDBF69; lane 2, pPFT2As; lane 3, pPFT2Af; lane 4, pPFT2Asf. B: Lane 1, pWF53; lane 2, pPFT11As; lane 3, pPFT11At; lane 4, pPFT11Ast. Lane 5 in both panels shows molecular mass standards.

## 3. Results

### 3.1. Effect of cyt1AP/STAB-SD on Cry2A yield

To compare the amounts of Cry2A produced by the different constructs, the yield of each was compared with the yield obtained with the wild-type construct (pDBF69), which was assigned a value of 1 (Fig. 2A, lane 1). With the exception of this control, the expression of all *cry2A* constructs was driven by *cyt1A* promoters. In comparison to the control, constructs which contained the STAB-SD sequence pro-

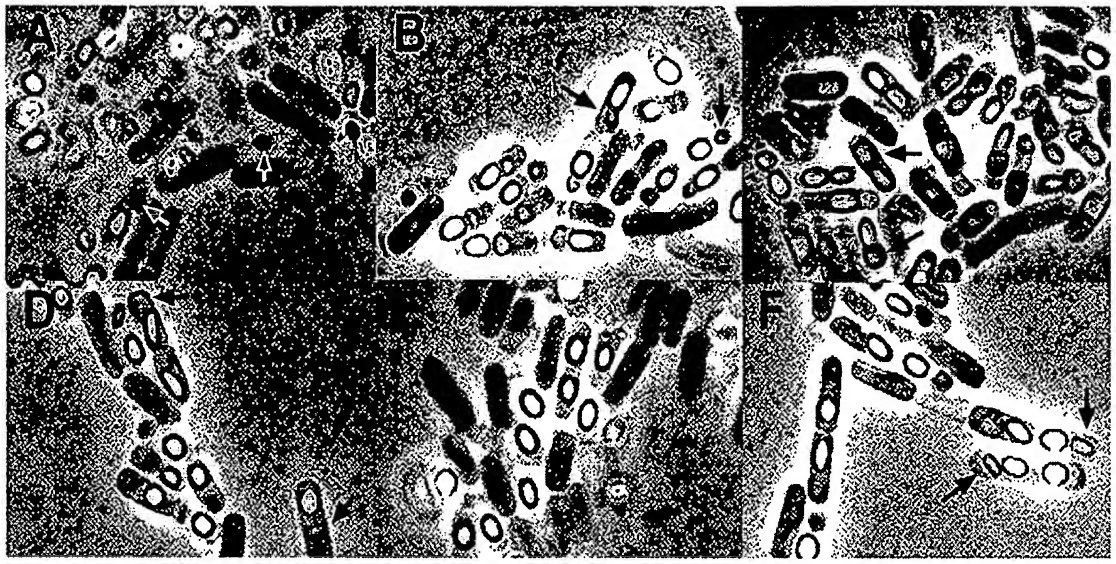


Fig. 3. Phase contrast micrographs of sporulated *B. thuringiensis* subsp. *israelensis* 4Q7 strains transformed with different *cry2A* and *cry11A* constructs. A: pDBF69; B: pPFT2Af; C: pPFT2Asf; D: pWF53; E: pPFT11At and F: pPFT11Ast. Arrows point to Cry2A or Cry11A crystals. The largest Cry2A (C) or Cry11A (F) crystals were produced by constructs with the STAB-SD sequence.

duced more Cry2A, but only produced typical cuboidal Cry2A crystals if the construct contained *orf2*, the gene encoding the scaffolding protein (Fig. 3A–C). The most significant increase in yield was obtained with the construct that contained both STAD-SD and *orf2* (Fig. 2A). Specifically, the construct pPFT2As, which contained the STAB-SD sequence but lacked *orf2*, produced 2.1-fold the yield obtained with the wild-type operon (Fig. 2A, lane 2). Despite this increase in yield, aggregates of Cry2A formed in cells, but no typical cuboidal crystals. The alternative construct pPFT2Af, which contained *orf2* but lacked the STAB-SD sequence, produced only 1.1-fold the amount of Cry2A obtained with the control (Fig. 2A, lane 3; Fig. 3A,B). In marked contrast to these results, pPFT2Asf, containing the STAB-SD sequence and *orf2* produced 4.4-fold the amount of Cry2A obtained with the control, and much larger crystals (Fig. 2A, lane 4; Fig. 3C). Yield increases based on an equal number of spores were higher. For example, the toxin yield for pPFT2Asf was six-fold higher than that of the control pDBF69 when the amount of toxin subjected to SDS-PAGE analysis was from an equivalent number of spores (data not shown).

### 3.2. Effect of cyt1AP/STAB-SD on Cry11A yield

The yield of Cry11A obtained when expression of its encoding gene was driven by *cyt1A* promoters combined with STAB-SD sequence increased significantly, but not nearly to the same extent as the yield obtained with Cry2A using the same expression system (Fig. 2B, lane 4; Fig. 3D–F). As in the Cry2A experiments, a value of 1 was assigned to the amount

Table 2
Final spore counts for eight different *B. thuringiensis* 4Q7 strains producing Cry2A or Cry11A

| Strain | Spores $ml^{-1} \times 10^8$ ($\pm \times 10^7$)[a] | | |
|---|---|---|---|
| *Cry2A constructs* | | | |
| pDBF69 | 8.03 | (2.08) | a |
| pPFT2As | 6.43 | (3.21) | b |
| pPFT2Af | 6.67 | (3.79) | b |
| pPFT2Asf | 7.33 | (1.53) | c |
| *Cry11A constructs* | | | |
| pWF53 | 4.07 | (2.52) | d |
| pPFT11As | 1.97 | (2.08) | e |
| pPFT11At | 2.40 | (3.61) | e |
| pPFT11Ast | 1.53 | (2.10) | e |

[a]Standard deviation. Values followed by different letters were significantly different at $P = 0.05$.

Table 3
Insecticidal activity of sporulated cell preparations or purified crystals of Cry2A or Cry11A

| Strain | $LC_{50}$ (95% fiducial limits)[a] | | | |
|---|---|---|---|---|
| | *T. ni* | | *A. aegypti* | |
| | Sporulated cells | Purified crystals | Sporulated cells | Purified crystals |
| 4Q7/pPFT2Asf | 1.78 (1.09-2.83) | 6.02 (0.16-11.9) | 44.25 (17.84-102.68) | > 500 |
| 4Q7/pDBF69 | 17.38 (13.05-23.20) | 34.86 (13.55–53.69) | 79.46 (34.34–274.67) | > 500 |
| 4Q7/pPFT11Ast | NT[b] | NT | NT | 73.0 (34.19–111.03) |
| 4Q7/pWF53 | NT | NT | NT | 152.79 (52.46–289.76) |

[a]All median lethal concentrations are presented as μg $ml^{-1}$ except $LC_{50}$ of purified Cry11A crystals against *A. aegypti* larvae (ng $ml^{-1}$).
[b]Not tested

of Cry11A synthesized by pWF53, a control construct similar to the wild-type operon (Fig. 2B, lane 1; Fig. 3D). The amount of Cry11A obtained with pPFT11Ast, which contained *cyt1A* promoters, the STAB-SD sequence and 20-kDa protein gene, was only 1.3-fold greater than the control (Fig. 2B, lane 4; Fig. 3F). Interestingly, no Cry11A was detected in the strains with the constructs lacking either the 20-kDa chaperone-like protein (pPFT11As; Fig. 2B, lane 2) or STAB-SD (pPFT11At; Fig. 2B, lane 3; Fig. 3E). As in the case of Cry2A, yield increases based on an equal number of spores were higher. The toxin yield for pPFTSAsf was 3.6-fold higher than that of the control pWF53 when the amount of toxin subjected to SDS-PAGE analysis was from an equivalent number of spores (data not shown).

### 3.3. *Effect of cyt1AP/STAB-SD on reproduction of 4Q7 cells*

The reproduction of the various Cry2A and Cry11A constructs on NBG based on spore counts is shown in Table 2. Reproduction of cells that expressed the *cry2A* constructs was higher than those expressing the *cry11A* constructs. For both toxins, the constructs which produced the highest toxin yields had lower spore counts than their respective controls, indicating that high toxin production decreased reproduction or the ability to produce viable spores. For Cry2A, this relationship was statistically significant, but the decrease in spore count amounted to only 9%. In the case of Cry11A, however, the decrease in spore count was greater than 60%, suggesting that the increase in toxin production inhibited reproduction or killed sporulating cells.

### 3.4. *Toxicity of crystal proteins produced using cyt1AP/STAB-SD*

The insecticidal activity of total cell lysates and purified crystals from the *cry2A* and *cry11A* constructs that produced the most toxin was tested against *T. ni* and *A. aegypti* larvae (Table 3). The 50% lethal concentration ($LC_{50}$) for Cry2A total cell lysates (pPFT2Asf in 4Q7) against *T. ni* larvae was 1.78 μg $ml^{-1}$ diet, approximately 10-fold higher than that obtained for cells transformed with the wild-type operon (pDBF69 in 4Q7) against *T. ni* larvae. In addition, both cell lysates were toxic to *A. aegypti* larvae, although the toxicity was quite low.

Against *T. ni*, purified Cry2A crystals from 4Q7/pPFT2Asf were more than five-fold as toxic as those produced by pDBF69 ($LC_{50}$ for = 6.02 versus 34.86 μg $ml^{-1}$). However, Cry2A crystals from either strain were not toxic to the first instar *A. aegypti*, even at the high concentration of 500 μg $ml^{-1}$ (Table 3). For Cry11A, the $LC_{50}$ of purified crystals produced by pPFT11Ast were slightly more than two-fold as toxic as those produced by the control pWF53 ($LC_{50}$ = 73.0 versus 152.79 ng $ml^{-1}$).

## 4. Discussion

We have shown that *cyt1A* promoters in combination with the *cry3A* STAB-SD mRNA stabilizing sequence [12,13] can enhance Cry2A and Cry11A yields, but that the degree of enhancement varies with the specific protein being produced. In comparison to wild-type or similar constructs, the yield of

Cry2A increased almost five-fold, whereas the increase for Cry11A was only 1.3-fold. In a previous study, it was shown that this expression system increased the yield of Cry3A by as much as 10-fold in comparison to the wild-type strain, providing additional evidence that the type of protein affects yield [12].

The yield increases obtained with *cyt1A*P/STAB are likely due to higher gene expression resulting from the use of *cyt1A* promoters and especially to greater transcript stability conferred by the STAB-SD sequence [13]. The much higher yield of Cry2A than Cry11A obtained with this expression system probably results from the former protein being more stable during and immediately after synthesis, prior to crystallization. Evidence for the comparatively poor stability of Cry11A is found in its requirement for the chaperone-like 20-kDa protein, encoded by the *cry11A* operon, for efficient synthesis [9–11,18]. In this regard, the absence of the gene encoding the 20-kDa protein in pPFT11As could account for the lack of detectable Cry11A (Fig. 2B). The lack of detectable Cry11A with the pPFT11At construct, however, which contained the 20-kDa protein gene but lacked the STAB-SD sequence, is more enigmatic. Expression of this construct was driven by the strong *cyt1A* promoters and it is possible that the higher levels of both Cry11A and the 20-kDa protein interfered with efficient synthesis of the former protein.

With respect to Cry2A synthesis, the inability of the strain harboring pPFT2As construct, which contained the STAB-SD sequence but lacked *orf2* of the *cry2A* operon, to produce typical Cry2A inclusions confirms several reports that ORF2 is essential for crystallization of Cry2A in *B. thuringiensis* [8,9,20]. ORF2 is not essential for Cry2A crystallization in *B. thuringiensis* subsp. *sotto* [21], but the amino acid sequence of this Cry2A varies by 4.6% from the sequence of the Cry2A of *B. thuringiensis* subsp. *kurstaki* HD1 [19], which may account for this difference.

An interesting finding of the present study is that the Cry2A crystals lacked toxicity to *A. aegypti*, though the toxicity of these crystals to *T. ni* was comparable to Cry2A crystals from *B. thuringiensis* subsp. *kurstaki* HD1 (Table 3). Results of several studies have shown that the mosquitocidal activity of *B. thuringiensis* subsp. *kurstaki* HD1 is due primarily to Cry2A [17,22]. This suggests that the mosquitocidal activity of Cry2A may be dependent on additional components in the spore/crystal mixture, as indicated by the lower $LC_{50}$ obtained using spore/crystal mixture of both 4Q7/pPFT2Asf and 4Q7/pDBF69. Alternatively, the *cyt1A*P/STAB system may have decreased the toxicity of Cry2A. In contrast to this, the toxicity of the Cry11A crystals produced using the *cyt1A*P/STAB system was two times that of the control. As these Cry11A crystals were much larger than control crystals, the increased toxicity was probably due to their more efficient ingestion by mosquito larvae.

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